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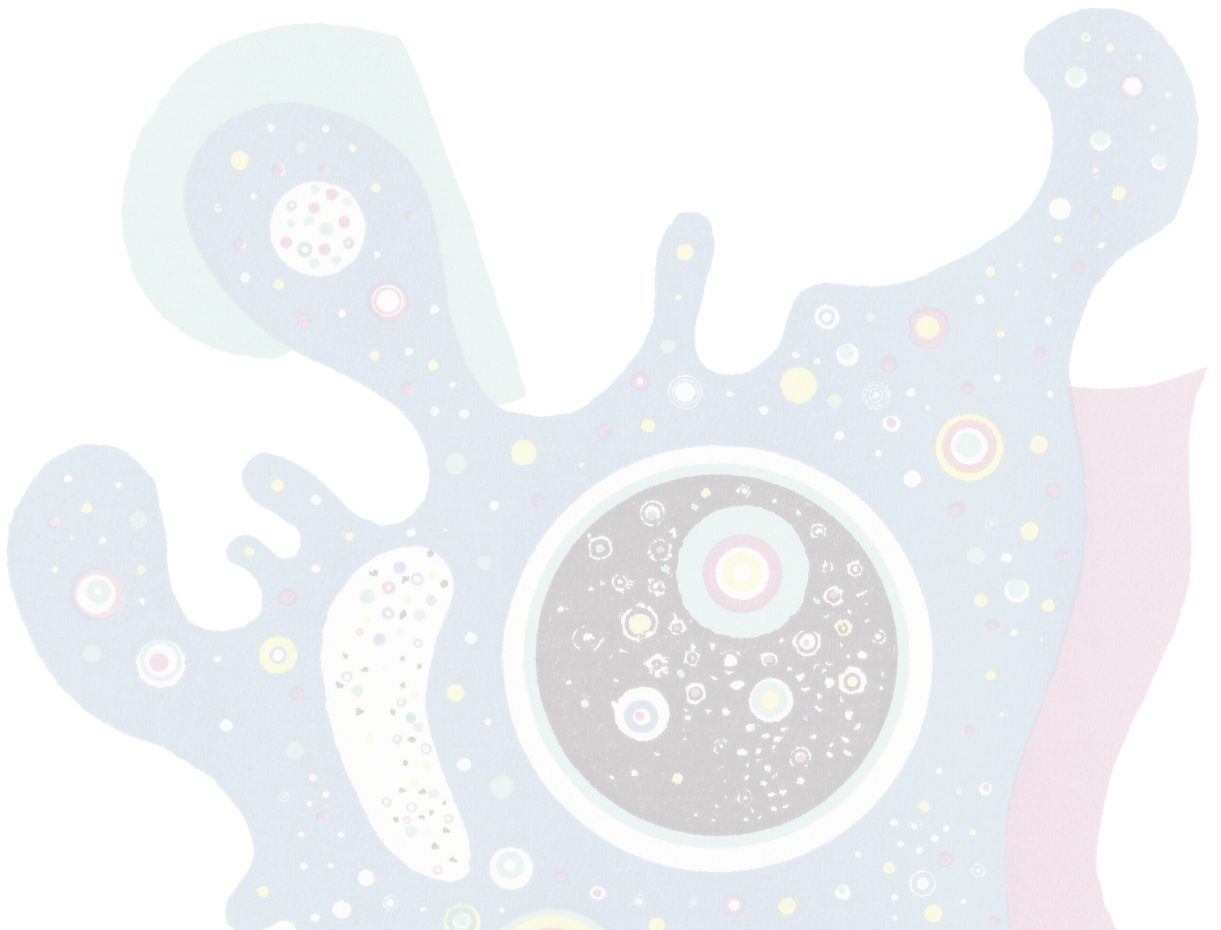
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Chapter 10

General discussion

Summary, conclusions and future perspectives



Neuronal degeneration and macrophage activation in Multiple Sclerosis

Neuronal degradation and axonal loss are key pathological features associated with disease progression and disability in Multiple Sclerosis (MS) ^{1,2}. Despite the strong association with clinical disease progression, the mechanisms of early axonal injury in MS are poorly understood ^{1,3}. Macrophages play a dominant role in the pathology of MS. MS lesion activity is characterized by demyelination in the presence of activated macrophages/microglia ⁴. Macrophage activity is related to axonal damage as exemplified by studies by Ferguson and colleagues who described accumulation of axonal amyloid precursor protein (APP), a marker for axonal dysfunction or injury, in active lesions and at the border of chronic active MS lesions ^{5,6}. Correlations between lesion activity and clinical disease progression support the idea that inflammatory mediators produced by macrophages/microglia play a role in tissue damage ⁵⁻⁷. Accumulating evidence suggests that oxidative stress and cytokines, produced by macrophages, are involved in axonal damage. Thus, prevention of oxidative stress and promotion of growth factors produced by macrophages may be successful strategies to prevent disease progression ⁸⁻¹³. To date, therapies applied for MS interfere with cellular/monocyte infiltration (anti-Very Late Antigen (VLA)-4; Natalizumab, interferon (IFN)- β), which prevents the formation of new lesions, but has limited effect on disease progression ¹⁴. However, it has been shown that macrophages can have both beneficial as well as damaging effects in neuroinflammatory diseases ¹⁵⁻¹⁷. Therefore, completely preventing monocytes to enter the central nervous system may have undesirable effects, since macrophages can also have beneficial effects on axonal repair. We hypothesized that the opposing effects of macrophages on disease progression could be due to their activation status. Two extreme activation phenotypes are the pro-inflammatory (M1) and the anti-inflammatory (M2) macrophages ^{18,19}. M1 macrophages produce large amounts of inflammatory mediators, including cytokines and reactive oxygen species (ROS) ^{20,21}, whereas M2 macrophages secrete growth factors, neurotrophic factors and cytokines associated with dampening inflammation ^{19,22-24}.

Aim of the thesis

The aim of this thesis was to elucidate the role of differentially activated human macrophages/microglia in relation to axonal damage and repair. In figure 1 we illustrate our hypothesis on how macrophages activation could play a role in MS lesion formation, having both a negative effect on disease progression through involvement in demyelination and axonal damage, and a beneficial role by promoting remyelination and axonal outgrowth.

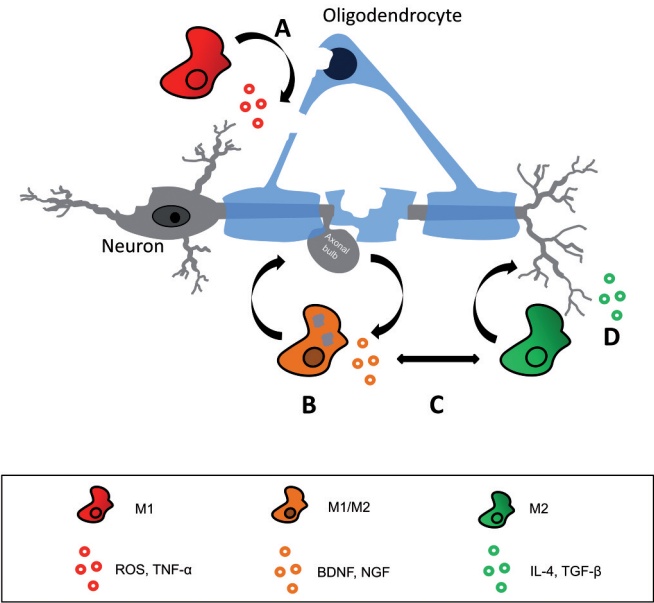


Figure 1. Proposed model of the role of macrophages in MS

In MS macrophages are recruited towards the CNS, and there are thought to play a key role in both damage and repair. It is widely assumed that in MS the macrophages in the CNS take on a pro-inflammatory state (M1) producing reactive oxygen species (ROS) and TNF- α (Chapter 2, 8) (A). In reaction to damage to myelin, these macrophages will phagocytose myelin debris, which increases neuronal repair and differentiates the macrophages into an intermediate activation status (Chapter 4) (B). All macrophages/microglia can switch between M1 and M2 and vice versa as shown in chapter 2 (C). Stressed (demyelinated) neurons produce Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), an important factor that stimulates the macrophages and microglia to an intermediate activation status (Chapter 3, 4 and 8). A key finding in this thesis is that GM-CSF also plays an important role in stimulating monocytes to cross the blood-brain barrier (Chapter 8). Upon damage, influx of M1 macrophages occurs, followed by recruitment of M2 macrophages. The M2 macrophages aid repair by secretion of anti-inflammatory cytokines such as TGF- β (Chapter 2 and 9)(D).

In this chapter, the findings of this thesis are summarized and important results and relevant findings are highlighted, and suggestions are made for future research.

Results

Polarization of human macrophages (Chapter 2)

Macrophages are key modulators and effector cells in the immune system. Their function differs depending on their activation status. Two activation phenotypes are extensively described; M1 (pro-inflammatory macrophages) and M2 (anti-inflammatory macrophages)^{18,19}. Many *in vitro* studies have established markers for rodent M1 and M2 macrophages^{25–28}, however few studies focus on human monocyte derived macrophages^{20,21,29,30}. The expression of markers and cytokines by rodent M1 and M2 macrophages is somewhat different from that in human (polarized) macrophages³¹. Furthermore, the methods of maturation and activation into M1 and M2 differ between research groups, making it difficult to compare data. In chapter 2 we compared the most commonly used maturation and activation protocols to determine the most distinctive markers for human M1 and M2 macrophages. We showed that CD40 and CD64 are most commonly expressed by M1 and CD163 and MR by M2. To study macrophage activation, *in vitro* studies have been crucial. However, classifying the macrophage activation status into M1 and M2 *in vitro* is an oversimplification of what may occur *in vivo* during disease. *In vivo* macrophages are influenced by a plethora of factors that are difficult to mimic in *in vitro* studies due to the many combinations of factors present in disease³². Nevertheless, the markers for M1 and M2 obtained by *in vitro* studies can be used as an indication for an M1 or M2 profile in the analysis of inflammatory infiltrates in tissues, including the CNS. The knowledge obtained in this study is also applicable to other inflammatory conditions.

The role for the M1 and M2 macrophages and microglia in lesion formation in MS (Chapter 3 and 4)

M1 macrophages/microglia are found throughout the CNS in mice and humans. It is widely assumed that the presence of the M1 macrophages hampers repair, regeneration and induces neurodegeneration while M2 macrophages/microglia are neuroprotective and stimulate repair^{15–17,33–35}. Due to these opposing functions of M1 and M2 macrophages, the activation status of macrophages/microglia might well play a role in lesion formation in MS. We examined the activation status of macrophages/microglia in preactive, active, chronic active and remyelinating lesions and compared the activation status to control brain tissue.

In control brain tissue, M1 markers (CD40, CD86 and CD74) are present on microglia and M2 markers are observed on macrophages in the perivascular space. In

active demyelinating lesions, the majority of the macrophages contained myelin and most macrophages express M1 markers. However, 70% of these macrophages also expressed M2 markers CD163 and MR, indicating an intermediate activation status.

In MS, clusters of microglia (preactive lesions) are observed in the normal appearing white matter (NAWM) and are considered to represent the first abnormality in MS brain. Preactive lesions are found in NAWM, before breakdown of the blood-brain barrier or demyelination can be detected³⁶. The high number of preactive lesions suggests that not all preactive lesions progress to active lesions, thus many are thought to resolve. To investigate whether the activation status of microglia and macrophages could influence the progression of preactive lesions into active demyelinating lesions in MS, we used a panel of markers distinguishing the two activation statuses. In preactive lesions the majority of microglia express M1 markers as well as the M2 cytokine CCL22, indicating an intermediate activation status (Chapter 4).

In chronic active MS lesions, macrophages positive for M2 marker expression were restricted to the perivascular space. In remyelinating lesions, the same marker expression as in preactive lesions was observed, the M2 markers were restricted to the perivascular space and M1 markers (CD40, CD86, CD74 and CCR7) together with M2 cytokine CCL22 were expressed. This indicates that in newly developing lesions (preactive) and remyelinating late lesions the activation status of macrophages/microglia is similar. This is in contrast with our hypothesis. We expected M1 macrophages to be predominant in early and active lesions, and M2 macrophages to be associated with repair and remyelination.

In the NAWM, accumulation of macrophages expressing membrane bound M2 markers (CD200R, MR and CD163) is only observed in the perivascular space, while limited expression of these markers is detected in the preactive lesions. However, in the active lesion, the expression of these M2 markers is observed on the myelin laden and perivascular macrophages, which suggests that myelin phagocytosis changes the activation status of the macrophage. It has been reported *in vitro* that myelin ingestion by macrophages skews the macrophages towards an anti-inflammatory activation status³⁵. In contrast, data from literature show that myelin ingestion results in the production of pro-inflammatory mediators^{37,38}. The mechanisms by which myelin affects the phenotype of macrophages and how this phenotype influences lesion progression remain unclear. Myelin modulates the phenotype of macrophages by peroxisome proliferator-activated receptor (PPAR) activation, which leads to an anti-inflammatory activation state with MR expression, which may subsequently dampen MS lesion

progression^{39,40}. It remains unclear whether the myelin-laden macrophages go into apoptosis after phagocytosis of myelin debris or migrate to the blood stream/perivascular space as suggested by the accumulation of M2 macrophages in the perivascular space in MS. There, the myelin laden macrophages could present myelin antigens to T-cells.

In experimental autoimmune encephalomyelitis (EAE), a model of MS, M1 macrophages/microglia are found throughout the CNS whereas M2 macrophages are mostly found in the perivascular space, the meninges and the choroid plexus, similar to that observed in the CNS in healthy mice³⁴. The presence of M2 macrophages in the human choroid plexus and meninges has not been reported thus far. Our preliminary observations in MS spinal cord (not included in this thesis) showed more M2 macrophages around the central canal of the spinal cord, in the meninges and in the perivascular space than in control spinal cord. This finding supports other reports that during neuroinflammation (such as in EAE and MS), macrophages displaying M2 markers are abundantly present. However, these macrophages are mostly found outside the lesion areas or CNS parenchyma, but rather in supporting/transporting tissues like meninges, choroid plexus and perivascular spaces^{34,41}.

Still the question remains whether, during a relapse in MS, M2 macrophages are actively recruited towards the lesion or act from the perivascular space. The M2 macrophages might be recruited and triggered by an unknown factor to secrete anti-inflammatory, neuroprotective agents. This remains to be established in the human CNS.

Taken together, during the different lesion stages in MS, most macrophages/microglia do not display the two extreme M1 or M2 phenotypes that were described *in vitro*. Apparently the presence of a wide range of cytokines and other macrophage activation ligands in tissues leads to an activation status of macrophages that is a result of all these factors together, and not of one or two “extreme” activators, like LPS or IL4, used for *in vitro* studies. It could be questioned if these extremes will ever be found *in situ*, but the paradigm has certainly been useful to strengthen the insight and awareness about the multiple roles of macrophages in tissue damage and repair. Whether the intermediate phenotype is a result of a M1 to M2 transition, or reflects exposure to multiple activators with their own effect on marker expression, is hard to conclude from the “snapshot” pictures of MS lesions. The homogeneity in marker expression in different stages of MS lesions pleads for the latter, rather than for transition from M1 to M2 or the other way around. A third possibility still remains, that is that some macrophages in MS tissue are derived from other precursors than others. The perivascular and meningeal

macrophages may originate from patrolling monocytes, whereas the macrophages in the lesions may be so-called “monocyte-derived macrophages”, derived from classical monocytes. For microglial cells it has already been shown that they have “their own” local precursor, originally derived from the yolk sac.

M2 macrophages migrate in higher numbers towards chemokines relevant for MS than M1 (Chapter 5)

Limited data are available on the functional properties of differently activated (M1 or M2) macrophages in the human CNS. For both EAE and an animal model for spinal cord injury (SCI) it has been shown that M1 macrophages are recruited to the lesion site first, followed by the appearance of M2 macrophages^{17,33,42,43}. M2 macrophages are suggested to enter the CNS through the choroid plexus whereas M1 macrophages enter the CNS at the lesion site³⁴. This difference in entry site for M1 and M2 macrophages suggests that M1 and M2 macrophages have different migratory capacities. For mouse macrophages it is shown that M2 migrates in higher numbers to CXCL12 and neuronal conditioned medium⁴⁴. However differences in migratory capacities between M1 and M2 macrophages have not yet been studied for human M1 and M2. Therefore we studied the migratory capacities of human M1 and M2 macrophages in chapter 5. We show that M2 macrophages migrate in higher numbers towards CCL2, CCL5, CXCL10, CXCL12 and C1q compared to M1 macrophages, which appears to be dependent on the macrophage’s ability to form filopodia. Our findings that M2 macrophages migrate over longer distances than M1 macrophages would fit with the theories suggesting that M2 (precursor) macrophages enter the CNS via the choroid plexus, whereas M1 macrophages are recruited and activated at the lesion site¹⁷. Shechter and colleagues proposed that, using data derived from an animal model for SCI, the choroid plexus supports trafficking of ‘healing’ cells to remote traumatized CNS by serving as a selective and ‘phenotype-shaping gate’ for monocytes *en route* to the damaged parenchyma³⁴. Here we show that human macrophages with a growth promoting, anti-inflammatory M2 phenotype indeed have the capacity to migrate over long distances towards CNS chemokines. In contrast, M1 macrophages, with potential damaging properties, remain at the site of their M1 activation and do not migrate.

GM-CSF triggers monocyte migration across the blood-brain barrier (Chapter 6)

Infiltration of monocytes into the CNS is crucial for the onset and progression of EAE ^{45,46}. The granulocyte-macrophage colony-stimulating factor (GM-CSF) has been suggested to play an essential role in monocyte migration across the blood-brain barrier ⁴⁷. We show that GM-CSF enhances monocyte migration across the human blood-brain barrier (BBB) *in vitro*. In EAE mice, GM-CSF is produced by pathogenic Th17 cells ⁴⁷. In MS patients GM-CSF levels are elevated in serum and liquor ⁴⁸, however it is unclear which cells are responsible for the production of GM-CSF in the CNS of MS patients. We show that in MS patients, GM-CSF is highly expressed by microglia, neurons, astrocytes and perivascular macrophages in the brain rather than by T cells. Cells susceptible to stimulation by GM-CSF are neurons (in the rim of combined grey and white matter lesions), astrocytes, microglia and endothelial cells, since they all express the GM-CSF receptor. Surprisingly, macrophages did not express GM-CSF receptor. We have no explanation for this unexpected finding. In particular, we do not know whether this truly represents absence of the receptor or rather the inability to immunostain the receptor with currently methodology. We consider the latter possibility unlikely, since the method was working perfectly well for the other cell types. *In vitro*, GM-CSF stimulation of macrophages induced an intermediate activation status resembling the phenotype observed in active MS lesions (Chapter 4), which shows that they can respond to GM-CSF. Therefore GM-CSF could be a potential physiological environmental activator of macrophages in the CNS. It is still unclear whether the GM-CSF produced by microglia, neurons, astrocytes or perivascular macrophages is pathogenic and necessary for sustaining inflammation in MS patients. In EAE, the GM-CSF produced by microglia is not pathogenic ⁴⁷. For that reason further studies are required to examine whether the GM-CSF produced by microglia, neurons, astrocytes or perivascular macrophages is damaging or protective for neurons. Further studies are also required to establish if indeed macrophages in lesions can respond to GM-CSF, or if the pro-inflammatory effect of GM-CSF is mainly mediated by monocytes. Currently a phase1b trial of anti-GMCSF is running for MS patients. Completely blocking GM-CSF might have undesirable effects, since GM-CSF also shows neuroprotective capabilities ⁴⁹, and therefore could stimulate repair.

Spinal cord and optic nerve (Chapter 7 and 8)

The spinal cord and optic nerve of MS patients may reveal more information on mechanisms of neurodegeneration and neuronal repair than the brain. Their anatomical

structure is less complex than that of the brain. Both optic nerve and spinal cord offer the possibility to study large numbers of axons, and relate (subtle) axonal changes to changes in the myelin sheath and/or the presence of activated macrophages and microglia. In particular longitudinal sections of the optic nerve allow recognition of disturbed axonal transport in relation to changes in the myelin sheath (Chapter 8). Studies on ultrastructural level would be an interesting next step in this line of research.

A limited number of papers from one research group describe the pathology of MS lesions in the optic nerve and spinal cord ^{1,50,51}. We described the distribution of MS lesions and their stages in detail. We show that the lesions present in the spinal cord and optic nerve contain less foamy macrophages than lesions in the brain. Comparing brain and spinal cord pathology revealed that lesions within one patient are heterogeneous with respect to lesion activity, and that spinal cord lesions do not reflect lesion activity in the brain and vice versa. Additionally, MS lesions in spinal cord do not respect the anatomical borders of grey and white matter or the septa between the bundles in the optic nerve. In white matter brain lesions and in the white matter part of combined grey and white matter lesions, the degree of inflammation is more pronounced than in the grey matter ⁵². This is also observed in the spinal cord, however this difference in degree of inflammation between the white matter and grey matter is less pronounced. In the optic nerve, which is composed of white matter only, the degree of inflammation is also less prominent than in the white matter of the brain.

Both tissues, i.e. optic nerve and spinal cord, are in close contact with the leptomeninges and therefore with CSF. CSF contains antibodies directed against neuronal and myelin proteins and these antibodies could be involved in the pathology observed in MS ^{53,54}. If CSF contains a myelinotoxic factor, this may contribute to demyelination in regions close to CSF. However we did not observe many white matter lesions in close contact with the meninges in the spinal cord or optic nerve. It remains to be determined whether factors such as pathogenic antibodies or cytokines like TNF in the CSF contribute directly to the pathology and whether such factors can indeed penetrate the spinal cord/parenchyma.

From our findings in optic nerve, brain and spinal cord lesions, we conclude that lesion staging as proposed by De Groot et al. has elements that could be improved upon ⁴. Lesion staging is an interpretation of neuropathological findings on post-mortem material. Microglia clusters (preactive lesions) are for example not specific for MS. Microglial clusters/nodules are a well-known phenomenon in viral encephalitis and AIDS, in which perineuronal aggregation of activated microglia/macrophages – indicative of

neuronal phagocytosis – is often observed ⁵⁵. Therefore the term ‘microglial clusters’ is possibly more appropriate than the term ‘preactive’ lesion.

The distinction between active and non-active lesions is based on macrophage activity. Our observations in the optic nerve and spinal cord show that active lesions at these sites contain lower numbers of macrophages/activated microglia than active lesions in the white matter of the brain. One potential explanation for this could be a difference in “age” of the lesions. For example, MS lesion activity may occur at an earlier stage of the disease in the spinal cord than in the brain and therefore the activity is less post mortem. The time line of lesion stages is not as clear as suggested by many researchers, and may differ for different sites in the CNS. Another explanation could be that the glial environment in optic nerve and spinal cord differs from that in the brain and is more effective in reducing lesion activity.

Macrophage activation in relation to neuronal damage and repair

In EAE there is strong evidence that M1 macrophages are related to neuronal damage and M2 to neuronal repair ^{15,34}. In MS, this has not been extensively studied despite the association of macrophages with axonal damage in MS lesions ⁵. In MS lesions activated macrophages are correlated with increased growth-associated protein-43 (GAP-43) ⁵⁶. However the relation of the activation status of macrophages with axonal damage has not been investigated. An observation in MS tissue is that the M2 macrophages accumulate in the perivascular space compared to healthy brain tissue ^{41,57}. To investigate the role of M1 and M2 macrophages in relation to neuronal damage and repair, we set out to generate cells with features of human adult neurons out of the SH-SY5Y neuroblastoma cell line ^{58,59}. After differentiation with retinoic acid (RA), which induces vesicle transport in these cells, co-culture and live cell imaging with M0 (non-activated macrophages), M1 and M2 revealed that all three subtypes could actively destroy the network that was formed.

Microglia are known to be capable of phagocytosing living neurons ^{60–65}. However these *in vitro* studies are performed with the neuroblastoma cell line, which are tumour cells, potentially stimulating the macrophages to phagocytose these cells. To investigate whether M2 are correlated with axonal damage or repair, we performed immunohistochemistry in active spinal cord lesions, staining M2 macrophages with MR and axonal damage with SMI32. This revealed no correlation between M2 and damaged or healthy neurons (SMI31). Additionally, and opposing findings in active lesions in the brain, myelin laden macrophages in spinal cord lesions did not express MR. Unfortunately

M1 staining was not successful due to technical difficulties. In conclusion, our preliminary data suggest that phagocytosis is the main function of macrophages in our *in vitro* experiments, and that this role is independent of their activation status. Furthermore, we found no evidence in our model supporting a neuroprotective role of M2 macrophages.

Future perspectives

Macrophage/microglia polarization *in vitro*, translation to *in vivo*

To answer the question to what degree *in vitro* polarization of cells to M1 and M2 phenotypes truly reflects cell profiles in MS lesions, another approach is advised. First of all, in most studies the intrinsic factors that could induce an M1 or M2 phenotype, such as Macrophage Colony-Stimulating Factor (M-CSF), GM-CSF, IFN- γ , IL-4, transforming growth factor (TGF)- β etc. are compared to, for example, LPS. These stimulation methods have provided relevant information on *in vitro* activation of macrophages. However, the main challenge now is to translate these *in vitro* findings to information that is relevant for the *in vivo* situation. The presence of LPS or other TLR4 ligands in an *in vivo* situation needs further investigation. Only a handful of studies are currently available in which levels of cytokines present in NAWM compared to control brain tissue have been reported⁶⁶. More information on cytokines that could potentially activate macrophages/microglia can also be acquired through mRNA studies of the different lesion stages obtained by laser capture dissection.

Migration of monocytes and macrophages

To further study the migration and attraction of M1 and M2 to the injured CNS in humans, future research might be better off to focus on the precursors of macrophages, the monocytes. During relapses in MS, the balance between classical and non-classical, patrolling monocytes, the putative precursors of M1 and M2 macrophages⁶⁷, is changed towards an increased number of classical monocytes⁶⁸. This may be highly relevant for lesion formation in the CNS. The migratory capacities of these classical and non-classical monocyte precursors of M1 and M2 macrophages across the blood-brain barrier have not yet been investigated.

The use of *in vitro* models

To allow further translation and validation of our findings to the human setting, *in vitro* models that adequately reflect the human brain micro-environment, including intimate

contacts between various CNS cells are crucial ⁶⁹. Human brain slice cultures of grey and white matter areas from healthy controls may fulfil this requirement and may be valuable to study the effects of macrophages on 'healthy' CNS tissue. The slice cultures can also be treated with lysophosphatidyl choline to mimic myelin damage, and therefore lesion formation ⁷⁰. Subsequently, M1 or M2 macrophages could be added and their effect on neuronal damage and repair, as well as de- and remyelination, could be evaluated. These humanised models could be the key to translating findings from animal models and to explore whether M2 macrophages are able to stimulate neuroregeneration and remyelination.

Macrophage activation for neuroprotection

In animal studies it is clear that M2 macrophages have dampening effects on the CNS ^{16,17,71}. We were not able to replicate these findings in human M2 macrophages induced by our protocols (IL-4). More research should be performed to determine the most effective biological triggers to induce a neuroprotective phenotype in human macrophages. While the production of nerve growth factors is a critical read out in this system, it can also be used to identify unknown factors promoting axonal survival and outgrowth in the supernatant. A stable M2 phenotype can be induced through maturation with M-CSF and stimulation with IL-4, IL-10 and TGFβ ³⁰. However, it has not yet been investigated if this phenotype secretes high amounts of neurotrophic factors. Treatment with glucocorticoids reduces clinical signs of MS and *in vitro* is able to induce an anti-inflammatory activation status in macrophages and microglia ^{72,73}. However, further studies should be performed to investigate potential therapies that selectively promote a neuroprotective phenotype in the CNS.

Conclusion

Cultures of a single cell type as an *in vitro* model are unable to mimic the complex profiles observed in tissues. Macrophages can develop mixed phenotypes expressing both M1 and M2 markers in pathological conditions. Macrophages do not form stable subsets, rather their plasticity enables them to respond to a combination of factors in local microenvironment of organs and tissues. While the M1/M2 paradigm is a useful model, reassessment is required to translate this hypothesis into suitable targets for modulatory strategies, with beneficial effects on inflammatory diseases. Future research should therefore focus on functional readouts, like phagocytosis, endocytosis, chemotaxis, adhesion and secretion of cytokines and growth factors to better characterize macrophage functional phenotypes. To profit from the anti-inflammatory effects of M2 macrophages in the CNS, we should focus on creating stable neuroprotective M2 macrophages, either by stimulating the M2 macrophage present in the perivascular space to proliferate and migrate towards the lesion or by modulating the precursor monocytes to mature into M2 macrophages.

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